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Patent Office

Ottawa, Canada
K1A 0C9

(21)	(A1)	2,099,542
(22)		1993/07/02
(43)		1994/01/08

5,070,0/52

(51) INTL.CL.⁵ C07D-493/04

(19) (CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Photochemical Labelling of Nucleic Acids with Europium
Chelate Reagents and Their Use in Gene Probe Test
Systems

(72) Löbbberding, Antonius - Germany (Federal Republic of) ;
Mikhail, Gamal K. - Germany (Federal Republic of) ;
Springer, Wolfgang - Germany (Federal Republic of) ;
Hugl, Herbert - Germany (Federal Republic of) ;
Köcher, Jürgen - Germany (Federal Republic of) ;

(71) Bayer Aktiengesellschaft - Germany (Federal Republic of)
;

(30) (DE) P 42 22 255.9 1992/07/07

(57) 5 Claims

Notice: This application is as filed and may therefore contain an
incomplete specification.

Canada

CCA 3254 (10-92) 41 7530-21-936-3254

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Photochemical labelling of nucleic acids with europium chelate reagents and
their use in gene probe test systems

A b s t r a c t

The present invention concerns photochemical labelling reagents comprising a lanthanide ion-chelating structure and a furocoumarin derivative bound via a spacer. The labelling reagent can be used in gen diagnostic.

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Gene probe diagnostics is a method for the sequence-specific detection of DNA/RNA sequences. It is based on the hybridisation of the gene probe sequence with complementary sequence regions of the DNA/RNA to be detected
5 [J.A. Matthews, L.J. Kricka, Analytical Biochemistry 169, 1-25 (1988); U. Landegren, R. Kaiser, C.T. Caskey, L. Hood, Science 242, 229 (1988)].

Gene probe diagnostics makes possible the detection of infectious diseases and genetic defects. Prerequisites
10 for the broad application of gene probe diagnostics are adequate sensitivity of detection, simplicity in performance and the avoidance of radioactivity.

One variant of gene probe diagnostics proceeds by way of the direct photochemical labelling of the DNA/RNA to be
15 detected; subsequently hybridisation occurs to gene probes with complementary nucleic acid sequences [N. Dattagupta, P.M.M. Rae, E.D. Huguenel, E. Carlson, A. Lyga, J.S. Shapiro, J.P. Albarella, Analytical Biochemistry 177, 85 (1989); J.P. Albarella, R.L. Minegar,
20 W.L. Patterson, N. Dattagupta, E. Carlson, Nucleic Acids Research 17, 4293 (1989)].

Furocoumarins which are linked to biotin by way of suitable spacer molecules have been shown to be very suitable for the photobiotinylation of nucleic acids.
25 After hybridisation to a gene probe with a complementary

nucleic acid sequence, and a separation step, detection takes place, for example by addition of a complex of antibiotin-antibody or avidin or streptavidin with alkaline phosphatase. For the detection, a colour reaction, which is elicited by alkaline phosphatase, is carried out in an additional step [J.J. Leary, D.J. Brigati, D.C. Ward, Proc. Natl. Acad. Sci. USA 80, 4045-4059 (1983)].

A disadvantage of the detection system using biotin is the wide distribution of biotin in biological systems.

A possible alternative would be direct photolabelling of the DNA/RNA to be detected, for example using a fluorescent dye. However, this has been found not to be practicable under the conditions of the photoreaction, because of preferential energy wasting. In addition, a suitable label would have to be photoinert.

Surprisingly, lanthanide chelates which are linked to suitable furocoumarins by means of a spacer have been found to be suitable.

Lanthanide chelates, in particular europium chelates, are already being routinely employed in immunodiagnosics [P. Degan, A. Abbondandolo, G. Montagnoli, J. of Bioluminescence and Chemiluminescence 5, 207 (1990)]. A particular advantage of using them is the possibility of time-resolved measurement of fluorescent light. Their application in gene probe diagnostics has now also been

described for the first time [A. Oser, W.K. Roth, G. Valet, Nucleic Acids Res., 3, 1181 (1988)], though in this work labelling with europium chelate reagents takes place using a costly procedure. In addition, the use of europium chelate primers for the PCR reaction has been described [P. Dahlen, A. Iitiä, V.-M. Mikkala, P. Hurskainen, M. Kwiatkowski, Molecular and Cellular Probes 5, 143-149 (1991)].

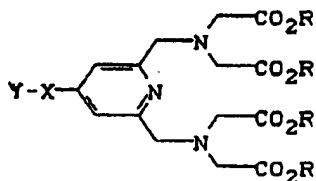
According to the invention, a labelling reagent of the general formula



is synthesised, where:

Ln = a lanthanide ion-chelating structure,
 S = a spacer molecule and
 Fu = a furocoumarin derivative as a photochemically linkable structure.

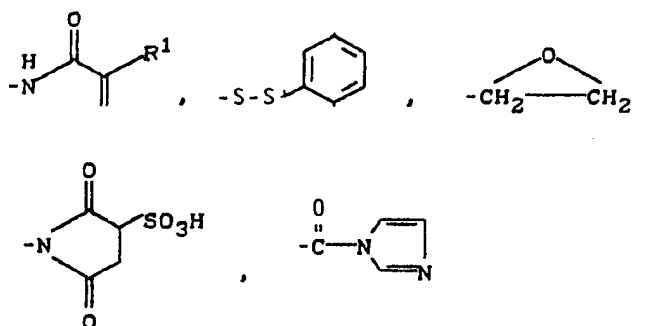
The lanthanide ion-chelating structure (Ln) is a pyridine derivative of the formula



where

X represents C_3 - to C_{14} -arylene, optionally containing a hetero atom grouping, or C_1 - to C_{24} -alkylene containing hetero atom groupings [N, O, S (1x, more than once)],

5 Y and optionally X + Y represents N-oxysuccinimido, N-maleimido, NH_2 , OH, $COCH_2$ -halogen, halogen, NCO, NCS, CHO, COOH, SH, CO-halogen, $COOCOR^1$, $CH=CHCO_2R^1$,



10 where R^1 represents hydrogen, a saturated or unsaturated C_1 - to C_{20} -alkyl radical, optionally substituted by a phenyl group, or a phenyl group,

R represents, in each case independently of the others, hydrogen, ammonium or an equivalent of an alkali metal or a 1/2 equivalent of an alkaline earth metal.

15

The synthesis of the pyridine derivative Ln takes place according to methods which are known per se [see, e.g., F. Vögtle and C. Ohm, Chem. Ber. 117, 849 to 854 (1984);

R. Singh and G. Just, J. Org. Chem. 54, 4453 (1989)].

The spacer is a polyalkylamine, a polyethylene glycol or a combination of these.

Polyalkylamines have the following general formula:



where

R represents H, C₁-C₇-alkyl, aryl (such as, e.g., phenyl, naphthyl or anthracyl), hydroxyl or C₁-C₇-alkoxy;

10 x represents a number between 2 and 7;

y represents a number between 3 and 10.

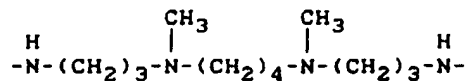
R can occur differently in the possible variants mentioned above, i.e. it must not be identical for each

15 repetition of the $\begin{array}{c} \text{R} \\ | \\ -(\text{CH}_2)_x-\text{N}- \end{array}$ unit in the spacer. The same is also the case for x, i.e. x must not be identical for each repetition of the $-(\text{CH}_2)_x-$ unit in the spacer.

20 Preferably the Rs, independently of each other, = H, C₁-C₄-alkyl (e.g. methyl, ethyl, n-propyl, i-propyl, n-butyl, i-butyl, tert-butyl); x = 2, 3, 4 or 5; and

y = 3, 4, 5 or 6.

Particularly preferred are N-4,N-9-dimethylspermine derivatives of the formula



- 5 Polyethylene glycols have the following general formula



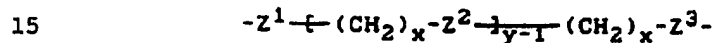
where

x is = 2, 3, 4 or 5 and

y is = 3, 4, 5 or 6.

- 10 Preferred are polyethylene glycols with x = 2, 3, 4 or 5; y = 3, 4, 5 or 6. Particularly preferred are polyethylene glycols with x = 2 and y = 4, 5 or 6.

Spacer molecules with combined amine/glycol structures have the following general formula:



where

Z¹, Z² and Z³, independently of each other, represent O or NR,

R represents H, C₁-C₇-alkyl, aryl (such as, e.g., phenyl, naphthyl or anthracyl), hydroxyl or C₁-C₇-alkoxy;

x represents a number between 2 and 7;

5 y represents a number between 3 and 10.

Preferred are spacer structures with $Z^2 = O$ and $Z^1, Z^2 = NR$ where

10 the Rs = H, C₁-C₄-alkyl (e.g. methyl, ethyl, n-propyl, n-butyl, i-butyl, tert-butyl); x = 2, 3, 4 or 5; and y = 3, 4, 5 or 6.

Particularly preferred are structures with $Z^2 = O$,
 $Z^1, Z^3 = NR$,

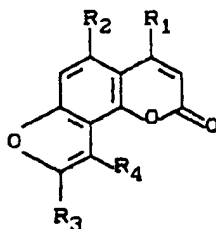
R = H, methyl, ethyl,

x = 2,

15 y = 6.

Suitable photochemically linkable structures are in particular furocoumarins, such as, for example, angelicin (isopsoralen) or psoralens as well as derivatives of these which react photochemically with nucleic acid.

Angelicin derivatives have the following general formula:



where

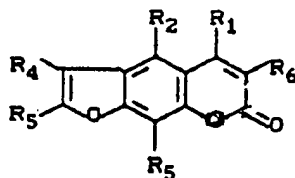
5 R_1 , R_2 and R_3 , independently of each other, represent H or C_1 - C_7 -alkyl, and R_4 represents H, C_1 - C_7 -alkyl or a lower alkyl with hydroxyl, C_1 - C_7 -alkoxy, amino, halo or N-phthalimido substituents.

Particularly preferred are angelicin derivatives which contain the following R_1 - R_4 groupings:

10	R_1	R_2	R_3	R_4
	H	H	H	H
	CH ₃	H	CH ₃	H
	CH ₃	CH ₃	CH ₃	CH ₂ OH
15	CH ₃	H	CH ₃	CH ₂ OCH ₃
	CH ₃	H	CH ₃	CH ₂ NH ₂
	H	H	CH ₃	CH ₂ Cl
	H	H	CH ₃	

Other compounds with different R's may also be synthesised by processes known from the literature.

Suitable psoralens have the following general formula:



5 where

R_1 , R_3 and R_5 , independently of each other, represent H or C_1-C_7 -alkyl,

10 R_4 represents H, C_1-C_7 -alkyl or C_1-C_7 -alkyl with hydroxyl, C_1-C_7 -alkoxy, amino, halo or N-phthalimido substituents,

R_2 and R_3 , independently of each other, represent H, hydroxyl, carboxyl, carbo- C_1-C_7 -alkoxy or C_1-C_7 -alkoxy.

15 Angelicin derivatives are advantageous in comparison with psoralens because of the monoadduct formation.

The sequence of the binding of the lanthanide ion chelating agent, the spacer and the furocoumarin is arbitrary. It is thus possible, inter alia, first to link the chelating agent Ln with the spacer S and subsequently to

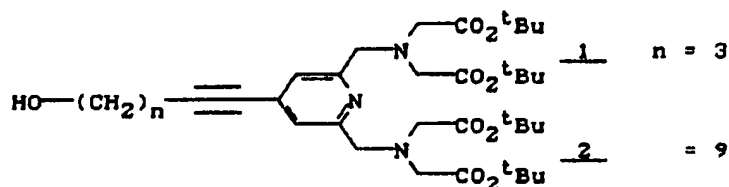
react the product with the furocoumarin Fu. Conversely, Fu-S may first be constructed and then reacted with Ln.

The linking of the moieties is effected in a manner known per se.

5 Examples

Example 1a)

Preparation of 2,6-bis[N,N-bis(t-butoxycarbonylmethyl)-amino-methyl]-4-(5-hoxypent-1-ynyl)pyridine (1):



- 10 6 g (10 mmol) of 2,6-bis[N,N-bis(t-butoxycarbonylmethyl)-amino-methyl]-4-bromopyridine (prepared as described by H. Tokalo, P. Pasanen and J. Kaukare in Acta Chem. Scand. Ser. B 42, (1988) 373) are dissolved in a mixture of freshly distilled tetrahydrofuran, 15 ml, and 15 ml of triethylamine. The solution is degassed and 1 g (12 mmol)
- 15 of 5-hoxypent-1-ine is introduced. The catalyst, consisting of a mixture of 280 mg (0.4 mmol) of bis(triphenylphosphine)palladium(II) chloride, 840 mg (3.2 mmol) of triphenylphosphine and 117 mg (0.61 mmol) of Cu(I) iodide, is added at room temperature and with stirring.
- 20 The reaction is complete according to TLC after 7 hours

of refluxing. After cooling to room temperature and subsequent filtration, the solution is concentrated in vacuo and chromatographed over silica gel (eluent: ethyl acetate, $R_f = 0.61$).

- 5 4.6 g (68% of theory) are obtained of a slightly yellowish solid with a melting point of 90°C.

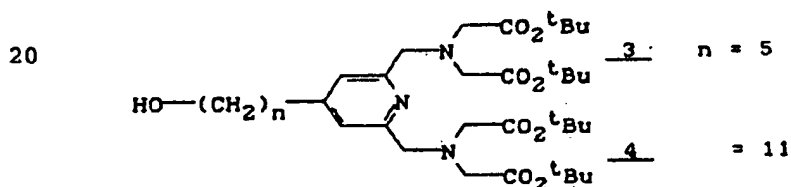
Example 1b)

Preparation of 2,6-bis[N,N-bis(t-butoxycarbonylmethyl)-amino-methyl]-4-(11-hydroxyundec-1-ynyl)pyridine (2):

- 10 6 g (10 mmol) of 2,6-bis[N,N-bis(t-butoxycarbonylmethyl)-amino-methyl]-4-bromopyridine are reacted with 1.93 g (12 mmol) of 1-undecyn-10-ol under the action of Pd catalysis in analogy with Example 1a). After chromatography on silica gel (eluent: ethyl acetate, $R_f = 0.52$),
15 7 g (77% of theory) are obtained of a yellow solid with a melting point of 57 to 59°C.

Example 2a)

Preparation of 2,6-bis[N,N-bis(t-butoxycarbonylmethyl)-amino-methyl]-4-(5-hydroxypentyl)pyridine (3):



472 mg (0.7 mmol) of the compound 1 described in Example 1 are dissolved in 20 ml of abs. ethanol and 24 mg of 10% Pd/C are added. The solution is vigorously stirred at 45 to 50°C under positive hydrogen pressure. The reaction is complete within 1 hour (according to TLC). After cooling and removal of the catalyst, the solution is concentrated in vacuo and the residue is chromatographed on silica gel (eluent: ethyl acetate, $R_f = 0.52$). 242 mg (51% of theory) are obtained of a slightly yellowish oil.

10 An improvement of the yield (56% of theory) is achieved if PtO_2 is used as the catalyst under the same reaction conditions.

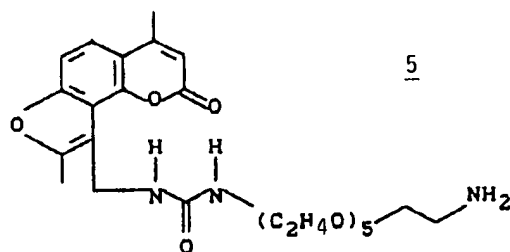
Example 2b)

15 Preparation of 2,6-bis[N,N-bis(t-butoxycarbonylmethyl)-amino-methyl-4-(11-hydroxyundecyl)pyridine (4):

1.0 g (1.32 mmol) of the compound 2 described in Example 1b) is hydrogenated with PtO_2 catalysis (100 mg) in analogy with Example 2a). After chromatography on silica gel (eluent: chloroform/ethanol 15 : 1, $R_f = 0.4$), 725 mg (72% of theory) are obtained of a slightly yellowish oil.

Example 3

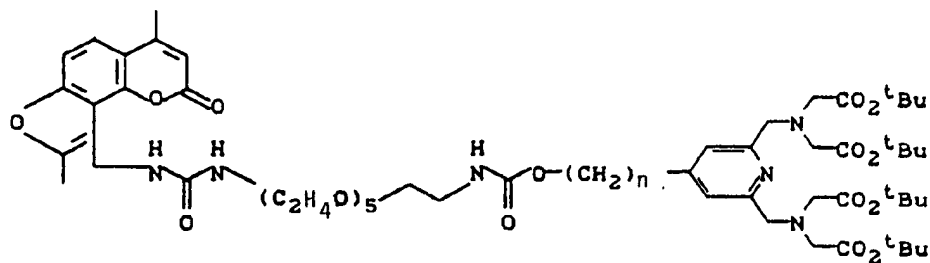
Preparation of amino-PEG-angelicin (5)



4.87 g (20 mmol) of 4'-aminomethyl-4,5'-dimethylangelicin are dissolved in 25 ml of DMF and reacted with 3.24 g (20 ml) of carbonyldiimidazole at room temperature. Complete reaction (according to TLC) was observed after 6 hours of stirring under nitrogen. The solution is slowly added dropwise to a solution of 16.85 g (60 mmol) of 1,17-diamino-3,6,9,12,15-pentaoxaheptadecane in 40 ml of DMF at 80°C and the mixture stirred at 70°C for a further 12 hours. After cooling, the solution is concentrated in vacuo and chromatographed on silica gel (eluent: chloroform/methanol/ammonia 90:10:1, $R_f = 0.28$). 7.1 g (65% of theory) are obtained of a slightly yellow oil.

Example 4a)

Preparation of Ln-S-Fn ester (6):

6 $n = 5$ 7 $n = 11$

- 250 mg (0.37 mmol) of the compound 3 described in Example 2a) are dissolved in 3 ml of dry toluene. 65 mg (0.4 mmol) of carbonyldiimidazole are added. After 17 hours of stirring at 60°C under N₂, 3 is completely reacted (according to TLC, eluent: chloroform/ethanol 15:1, R_f = 0.45) and a new product has formed (eluent: see above, R_f = 0.63). 220 mg (0.4 mmol) of the compound 5 described in Example 3 are added and the reaction mixture is stirred at 90°C for a further 24 hours. After cooling, the solution is concentrated in vacuo and the residue is chromatographed on silica gel (eluent: toluene/ethanol 5 : 1, R_f = 0.36). 138 mg (30% of theory) are obtained of a slightly yellow oil.

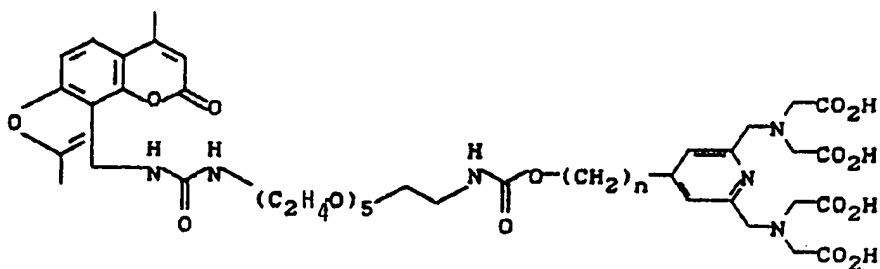
Example 4b)

Preparation of Ln-S-Fn ester (7):

410 mg (0.54 mmol) of the compound 4 described in Example 2b) are activated with carbonyldiimidazole and subsequently reacted in analogy to Example 4a) with the amino-PEG-angelicin 5 described in Example 3). After chromatography on silica gel (eluent: toluene/ethanol 5:1, $R_f = 0.31$), 188 mg (26% of theory) are obtained of a yellowish oil.

10 Example 5a)

Preparation of Ln-S-Fn-tetracarboxylic acid (8):

8 $n = 5$ 9 $= 11$

138 mg (0.11 mmol) of the tetraester 6 described in Example 4a) are dissolved in 4 ml of dry benzene and 569 mg (5 mmol) of trifluoroacetic acid are added under N_2 . After 2 hours of stirring at 60°C, the product

separates out in benzene as an oil. According to TLC the reaction is complete. After cooling, the solution is concentrated in vacuo. The residue is dissolved in 5 ml of distilled water and extracted by shaking twice with 3 ml of diethyl ether. The aqueous phase is concentrated and chromatographed on RP 18 (eluent: methanol, $R_f = 0.13$). 70 mg (62% of theory) are obtained of a milky, viscous oil.

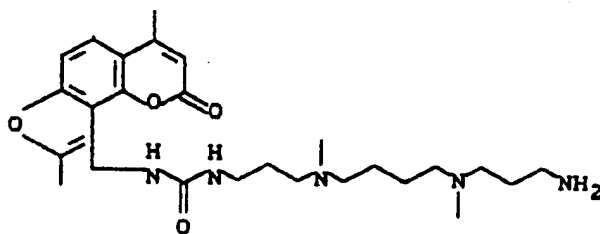
Example 5b)

10 Preparation of Ln-S-Fn tetraacid (9):

45 mg (0.034 mmol) of the tetraester 9 described in Example 4b) are reacted with trifluoroacetic acid in analogy with Example 5a). 30 mg (81% of theory) are obtained of a viscous oil.

15 Example 6

Preparation of N^1 -(angelicinamido)- N^4, N^8 -dimethylspermine (10):

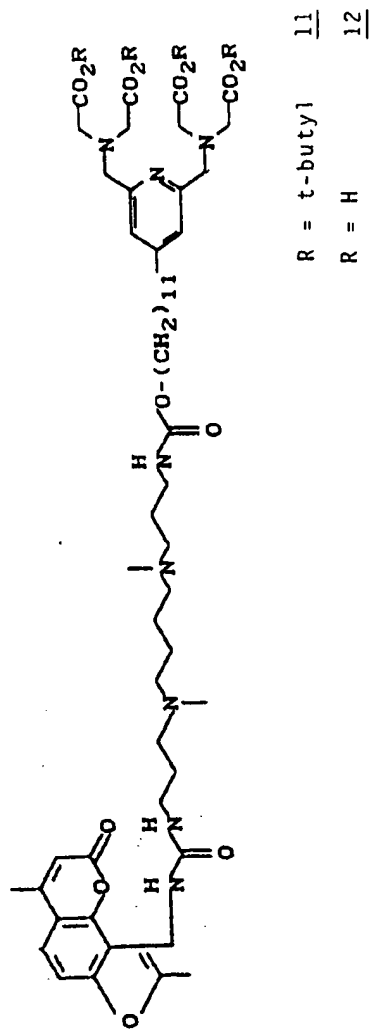


10

- 5 4.87 g (20 mmol) of 4'-aminomethyl-4,5'-dimethylangelicin are activated with carbonyldiimidazole in analogy with Example 1. The resulting solution is added dropwise to a solution of 13.8 g (60 mmol) of N⁴,N⁹-dimethylspermine in 40 ml of DMF in analogy with Example 1. After cooling, the solution is concentrated in vacuo and the residue is chromatographed on silica gel (eluent: chloroform/methanol/ammonia 30:5:1, R_f = 0.11). 7.1 g (71% of theory) are obtained of a yellow oil.

Example 7

Preparation of Ln-S-Fn ester (11):



- 318 mg (0.5 mmol) of the compound 4 described in Example 2b are activated with carbonyldiimidazole and subsequently reacted with the amino compound 10 described in Example 6 in analogy with Example 4. After chromatography on silica gel (eluent: chloroform/methanol/ammonia 70:45:1, $R_f = 0.42$), 132 mg (21% of theory) are obtained of a yellow oil.

Example 8

Preparation of Ln-S-Fn tetraacid (12): (EuPA)

- 10 30 mg (0.023 mmol) of the tetraester 11 described in Example 7 are reacted with trifluoroacetic acid in analogy with Example 5. 22 mg (92% of theory) are obtained of a yellow oil.

Example 9

- 15 Photoreaction of hairpin oligonucleotides with EuPA (12)

- 50 μ g of the hairpin oligonucleotide are taken up in 100 μ l of Tris-HCl buffer. The solution is left in the waterbath at 50°C for 15 minutes. To permit slow cooling to room temperature, the sample is taken out of the waterbath. Subsequently a further 400 μ l of water are added.

For the photoreaction, a 20-fold molar excess of EuPA is added to 15 μ g of the hybridised hairpin oligonucleotide.

The solution is subsequently illuminated under a UV lamp at 312 nm or 366 nm in an Eppendorf tube. The photo-reaction is followed using HPLC. Within 15 minutes, the photoreaction was complete.

5 Example 10

Photolabelling with EuPA (12)

10 For the photolabelling, 50 μ l of 1 M sodium tetraborate buffer pH 8.3 and 50 μ l of EuPA (2 μ g/ μ l) were added to 2 to 5 μ g of DNA in 20 μ l of TE buffer and the solution made up to 500 μ l with double-distilled H₂O. The mixture was irradiated for 10 minutes at 312 nm using a UV trans-illuminator, with the samples being kept on ice during this period.

15 The photolabelled DNA was subsequently precipitated with 1/10 volume of 3 M sodium acetate pH 5.8 and 1 volume of isopropanol at room temperature and left to stand for 5 minutes. Subsequently, the DNA was centrifuged down at 10,000 rpm in an Eppendorf centrifuge, the supernatant decanted off and the DNA precipitate washed with 70%
20 strength ethanol. After the samples had been dried, the photolabelled DNA was taken up in TE. The photolabelling of the DNA with EuPA was subsequently monitored by agarose gel electrophoresis and microtitre tests.

Example 11

Detection of the EuPA labelling in the microtitre test

5 To detect the EuPA labelling of double-stranded DNA, the
DNA was pipetted, after the labelling, into microtitre
test plates in concentrations of 250 ng to 125 pg in 1:2
dilution steps. To attach the DNA to the polystyrene
groups of the microtitre wells, the DNA was first diluted
10 in the wells with PBSM buffer (10 mM Na phosphate pH 7.2
with 0.1 M MgCl₂, 0.15 M NaCl, 3 M KCl) and incubated at
room temperature overnight. Washing 2x with 200 µl of
PBSM buffer subsequently took place, and the DNA was
fixed to the wells by 10-minute irradiation with a UV
transilluminator at 312 nm. The DNA fixed in this way was
subsequently washed 4x with wash-concentrate buffer from
15 Delfia/Pharmacia, in order to remove excess EuPA loaded
with europium. As the negative control, unlabelled,
double-stranded DNA was treated in the same manner.

Following addition of 100 µl of enhancement solution from
Wallac/Pharmacia, the time-resolved fluorescence of
20 europium was measured, after 30 minutes at room tempera-
ture, in a DELFIA 1232 fluorescence photometer from
Wallac/ Pharmacia at 290 to 360 nm excitation/615 nm
emission. Depending on the dilution of the DNA, fluore-
scence signals of 212,000 to 1,700 were measured in the
25 labelled DNA. The unlabelled DNA only gave low background
signals.

Example 12

Hybridisation of EuPA-labelled genomic DNA in the reversed phase test

5 The preparation of EuPA-labelled DNA was carried out according to the method described in Example 10.

10 The hybridisation was carried out by conventional processes at an incubation temperature of 40 to 68°C. Different substances were added depending on the hybridisation temperature. With long gene probes, dextran sulphate or other polymers were employed in order to increase the speed and extent of the hybridisation. Detergents and blocking reagents, such as dried milk, Denhardt's solution, heparin or SDS, were employed in order to suppress the non-specific binding of the DNA to
15 the membrane. Denaturing agents, such as urea or formamide, may be employed in order to reduce the melting temperature of the hybrid, so that lower hybridisation temperatures may be used. In addition, the non-specific binding of probes to non-homologous DNA on the blot may
20 be reduced by the addition of heterologous DNA.

To prepare for the hybridisation, 100 ng of the unlabelled E.coli-specific gene probes (1.7 kb to 6 kb) were first denatured at 100°C for 5 minutes, cooled to 0°C, and then transferred to pre-treated nitrocellulose
25 or nylon membranes using a Minifold-II filtration apparatus from Schleicher and Schüll and fixed at 80°C

for 2 hours.

5 The filters were hybridised in a sealed plastic film bag or plastic box with at least 20 ml of hybridisation solution per 100 cm² of filter at 68°C for at least 1 hour.

10 The solution was replaced by 2.5 ml of hybridisation solution of 100 cm² of filter to which solution freshly denatured (100°C, 5 minutes), EuPA-labelled, genomic DNA from E.coli (1 µg) had been added. The filters were incubated at 68°C for at least 6 hours with gentle shaking.

15 The filters were then washed 2 x 5 minutes at room temperature with at least 50 ml of 2xSSC, 0.1% SDS per 100 cm² of filter and 2 x 15 minutes at 68°C with 0.1xSSC, 0.1% SDS.

20 The filters were then directly employed for detecting the hybridised DNA. Depending on whether EuPA-DNA was used which was already loaded with europium, or which was subsequently loaded with europium, the following further steps were carried out in working up the filters for the fluorescence read-out. In the case of EuPA-labelled genomic DNA which was not loaded with europium, the filters were treated in 100 µM EuCl₃, 100 µM EDTA and 1xSSC pH 7.0 in a total volume of 2 ml at room temperature for 2 hours. The filters were then washed six times
25 with 2xSSC. Subsequently, the individual slots of the

hybridisation blot were cut out and treated with 1 ml of enhancement solution in 1.5 ml reaction tubes. After a 30-minute incubation at room temperature, 200 μ l of the samples from the individual slots were pipetted into microtitre plates and the samples were measured in a DELFIA 1232 fluorescence photometer from Wallac/Pharmacia at 290 to 360 nm excitation and 615 emission.

In the case of slot blots with EuPA-labelled DNA, which had been loaded with europium before the labelling, the individual slots were cut out directly after the hybridisation and 1 ml of enhancement solution was added to them in 1.5 ml reaction tubes and then, as described above, the enhancement solution was added and measurement took place in a fluorescence photometer.

15 Solutions:

20 \times SSC: 3M NaCl, 0.3 M Na citrate pH 7.0
 Hybridisation solution: 5 \times SSC; 0.1% N-lauroylsarcosine, Na salt, 0.02% SDS; 0.5% blocking reagent (Boehringer), dissolve the solution at 50 to 70°C.

Example 13

Hybridisation with EuPA-labelled gene probes

The preparation of EuPA-labelled gene probes (1.7 to

6 kb) was carried out according to the method described in Example 10.

5 The EuPA-labelled gene probes may be employed in solid-phase or liquid hybridisations. Suitable solid phases are, for example, nitrocellulose membranes, nylon membranes, polystyrene groups of microtitre plates or magnetic particles. The fluorescent hybridisation complexes of gene probes with complementary genomic DNA may be separated from free fluorescent gene probes using
10 hydroxyapatite.

For example, a slot-blot hybridisation was carried out with EuPA-labelled, E.coli-specific gene probes (1.7 kb to 6 kb) and genomic DNA from E.coli.

15 For this purpose, the genomic E.coli DNA was denatured at 100°C for 5 minutes and then cooled to 0°C and then transferred to nitrocellulose or nylon membranes using a Minifold-II filtration apparatus from Schleicher and Schüll in the concentrations 500 ng to 125 pg in 1:2 dilution steps. The prehybridisation and hybridisation
20 were carried out as described in Example 12. 100 ng of EuPA-labelled E.coli gene probe were employed.

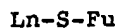
The read-out took place as described in Example 12 by individual measurement of the excised filter slots after treatment with enhancement solution in a DELFIA 1232
25 fluorimeter from Wallac/Pharmacia.

Using the gene probes, 125 ng of genomic DNA from E.coli were still readily detectable. This corresponds to a test sensitivity of 0.1 pg of DNA measured in the hybridisation of pure pBR322 plasmid probe to pBR322 DNA.

- 5 Alternatively, microtitre hybridisation tests were carried out. For this purpose, the genomic E.coli DNA was denatured as described above, and then diluted samples of 10 ng to 45 pg were pipetted into microtitre wells and left to stand at room temperature overnight. Subsequently, washing took place 2 x with 200 μ l of PBSM buffer and the DNA was then fixed for 10 minutes at 312 nm using a UV transilluminator. 200 μ l of hybridisation solution (Example 12) with 10 ng of EuPA-labelled gene probe were added and the hybridisation mixture was
- 10 incubated at 68°C for at least 6 hours. Subsequently, the microtitre wells were washed 2 x 5 minutes at room temperature with 2 x 200 μ l of 2 x SSC, 0.1 SDS and 2 x 15 minutes at 50°C with 2 x 200 μ l of 0.1 x SSC, 0.1% SDS.
- 15
- 20 The read-out took place as in Example 12 in a DELFIA 1232 fluorimeter from Wallac/Pharmacia after treatment of the wells with 100 μ l of enhancement solution.

Patent Claims

1. Labelling reagent of the general formula



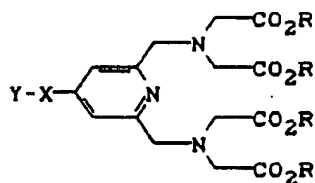
where

Ln is = a lanthanide ion-chelating structure,

S is = a spacer molecule and

Fu is = a furocoumarin derivative.

2. Labelling reagent according to Claim 1, where the lanthanide ion-chelating structure (Ln) is a pyridine derivative of the formula

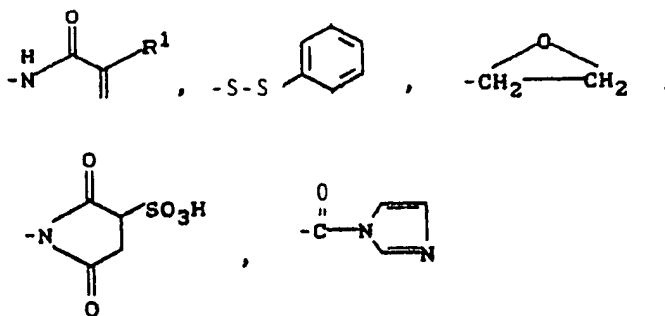


where

X represents C_3 - to C_{14} -arylene, optionally containing a hetero atom grouping, or C_1 - to C_{24} -alkylene containing hetero atom groupings [N, O, S (1x, more than once)],

Y and optionally X + Y represents N-oxysuccinimido, N-maleimido, NH_2 , OH, COCH_2 -halogen,

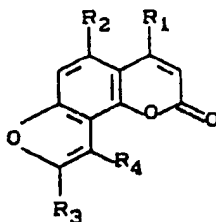
halogen, NCO, NCS, CHO, COOH, SH, CO-halogen,
COOCOR¹, CH=CHCO₂R¹,



where R¹ represents hydrogen, a saturated or unsaturated C₁- to C₂₀-alkyl radical, optionally substituted by a phenyl group, or a phenyl group,

R represents, in each case independently of the others, hydrogen, ammonium or an equivalent of an alkali metal or a 1/2 equivalent of an alkaline earth metal.

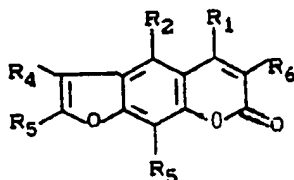
3. Labelling reagent according to Claim 1, where the spacer is a polyalkylamine, polyethylene glycol or a combination of these.
4. Labelling reagents according to Claim 1, where Fu is an angelicin derivative of the following general formula:



where

R_1 , R_2 and R_3 , independently of each other, represent H or C_1 - C_7 -alkyl, and R_4 represents H, C_1 - C_7 -alkyl or a lower alkyl with hydroxyl, C_1 - C_7 -alkoxy, amino, halo or N-phthalimido substituents.

5. Labelling reagent according to Claim 1, where Fu is a psoralen with the following general formula:



where

R_1 , R_3 and R_5 , independently of each other, represent H or C_1 - C_7 -alkyl,

R_4 represents H, C_1 - C_7 -alkyl or C_1 - C_7 -alkyl with hydroxyl, C_1 - C_7 -alkoxy, amino, halo or N-phthalimido substituents,

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R_2 and R_3 ,

independently of each other, represent H, hydroxyl, carboxyl, carbo- C_1 - C_7 -alkoxy or C_1 - C_7 -alkoxy.

Fetherstonburgh & Co.,
Ottawa, Canada
Patent Agents